THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 272, No. 21, Issue of May 23, pp. 13883–13891, 1997

Printed in U.S.A.

The Roles of Pteridine Reductase 1 and Dihydrofolate Reductase-Thymidylate Synthase in Pteridine Metabolism in the Protozoan Parasite *Leishmania major**

(Received for publication, August 15, 1996, and in revised form, December 30, 1996)

Bakela Nare, Larry W. Hardy‡, and Stephen M. Beverley§

From the Department of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, Boston, Massachusetts 02115 and ‡Department of Pharmacology and Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605

Trypanosomatid protozoans depend upon exogenous sources of pteridines (pterins or folates) for growth. A broad spectrum pteridine reductase (PTR1) was recently identified in *Leishmania major*, whose sequence places it in the short chain alcohol dehydrogenase protein family although its enzymatic activities resemble dihydrofolate reductases. The properties of PTR1 suggested a role in essential pteridine salvage as well as in antifolate resistance. To prove this, we have characterized further the properties and relative roles of PTR1 and dihydrofolate reductase-thymidylate synthase in *Leishmania* pteridine metabolism, using purified enzymes and knockout mutants. Recombinant *L. major* and *Leishmania tarentolae*, and native *L. major* PTR1s, were tetramers of 30-kDa subunits and showed similar catalytic properties with pterins and folates (pH dependence, substrate inhibition with H_2pteridines). Unlike PTR1, dihydrofolate reductase-thymidylate synthase showed weak activity with folate and no activity with pterins. Correspondingly, studies of *ptr1*$^-$ and *dhfr-ts*$^-$ mutants implicated only PTR1 in the ability of *L. major* to grow on a wide array of pterins. PTR1 exhibited 2000-fold less sensitivity to inhibition by methotrexate than dihydrofolate reductase-thymidylate synthase, suggesting several mechanisms by which PTR1 may compromise antifolate inhibition in wild-type *Leishmania* and lines bearing *PTR1* amplifications. We incorporate these results into a comprehensive model of pteridine metabolism and discuss its implications in chemotherapy of this important human pathogen.

Leishmania are trypanosomatid protozoan parasites that infect millions of people worldwide (1). Leishmaniasis takes several forms, ranging from minor or severe disfiguring cutaneous lesions to the deadly visceral form, depending upon the species and immune status of the host. Vaccines against *Leishmania* are not yet available, and treatment currently relies on the antiquated pentavalent antimonial compounds. These drugs are often toxic, sometimes ineffective, and their mode of action remains unknown. A better understanding of novel biochemical pathways of this primitive eukaryotic parasite clearly would be helpful in the development of selective anti-*Leishmania* drugs. For example, although antifolates are a mainstay in the treatment of parasitic diseases such as malaria, they have not proven clinically effective against *Leishmania* (2, 3). This may reflect the fact that *Leishmania* and related trypanosomatids exhibit a number of unusual features in pteridine (pterin and folate) metabolism. Improved knowledge of this pathway would likely allow the development of antifolates effective against this important disease.

Leishmania and other trypanosomatids including *Crithidia* are unable to synthesize the pterin moiety from GTP and thus must acquire pteridines from the host by salvage mechanisms (2, 4–11). This feature led historically to an appreciation of the pterin requirement of eukaryotes, where pterins are now known to participate as essential cofactors in hydroxylations, ether-lipid cleavage, and NO synthase (12–15). However, the pathways involved in the salvage and metabolism of pterins, and their function in *Leishmania,* are only beginning to emerge (9, 10).

Recently, we identified a novel pteridine reductase (PTR1)[1] in *Leishmania* (10). *PTR1* (formerly *hmtx*^{r} or *ltdh*) was originally identified as the gene responsible for methotrexate (MTX) resistance on the amplified H region in several species of *Leishmania* (16, 17). Sequence comparisons placed the predicted PTR1 protein in a large family of aldo-keto reductases and short chain dehydrogenases, a family including both dihydropteridine and sepiapterin reductases (16–19). The ability of PTR1 to reduce pteridines such as biopterin and folate was established by genetic and biochemical approaches in our laboratory (10). First, *ptr1*$^-$ null mutants specifically required H_2- or H_4biopterin for growth, a requirement not satisfied by H_2- or H_4folate. Second, partially purified recombinant PTR1 protein exhibited NADPH-dependent reductase activity with biopterin and folate and lesser activity with H_2biopterin or H_2folate (10). These properties placed PTR1 in a position to play a key role in the salvage of oxidized pterins. Moreover, the H_2folate reductase activity of PTR1, when combined with its relative insensitivity to MTX inhibition (100 nM *versus* 0.1 nM for DHFR-TS; Ref. 20), suggested that PTR1 could compromise antifolate inhibition of *Leishmania* (10).

Despite the homology of PTR1 to the short chain alcohol dehydrogenase superfamily (16–18), its enzymatic properties overlap those of many dihydrofolate reductases (DHFR), which is remarkable given their evolutionary divergence. The major

* This work was supported by grants from the National Institutes of Health (to S. M. B.) and the United States Public Health Service (to L. H.) and a postdoctoral fellowship from the Charles King Trust (to B. N.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Biological Chemistry and Molecular Pharmacology, Harvard Medical School, 240 Longwood Ave., Boston, MA 02115. Tel.: 617-432-0611; Fax: 617-432-1476; E-mail: sbeverle@warren.med.harvard.edu.

[1] The abbreviations used are: PTR1, pteridine reductase 1; MTX, methotrexate; DHFR-TS, dihydrofolate reductase-thymidylate synthase; H_2biopterin, dihydrobiopterin; H_4biopterin, tetrahydrobiopterin; H_2folate, dihydrofolate; H_4folate, tetrahydrofolate; Mes, 4 morpholinethanesulfonic acid; DHPR, dihydropteridine reductase.

role of DHFR is to convert H_2folate to the biochemically active H_4folate, a step needed for *de novo* synthesis of thymidylate, and in bacteria and higher eukaryotes, purine nucleotides (trypanosomatids are auxotrophic for purines). In *Leishmania* as well as all protozoans and plant species examined thus far, DHFR is part of a bifunctional polypeptide that also encodes thymidylate synthase (DHFR-TS; Refs. 21–23). Direct comparison of the enzymatic properties of PTR1 and DHFR-TS would help in the elucidation of the salvage and metabolism of pteridines in *Leishmania*. Additionally, such information could establish the suitability of PTR1 and/or DHFR-TS as targets for rational *Leishmania* chemotherapy.

Here we have purified both native and recombinant *L. major* PTR1s as well as recombinant *Leishmania tarentolae* PTR1, and have characterized their properties including K_m, V_{max}, pH dependence, and inhibition by substrate and MTX. Comparisons of the wild-type and *ptr1*$^-$ and *dhfr-ts*$^-$ knockout *Leishmania* showed that the ability to grow in diverse pterins correlated with their activity with PTR1 but not DHFR-TS, establishing PTR1 as the sole mediator of oxidized pterin salvage. Comparisons of the properties of PTR1 and DHFR-TS enzymes, and pteridine reductase activities in crude *Leishmania* extracts (including those from *ptr1*$^-$ and *dhfr-ts*$^-$ mutants), were used to establish the relative contribution of these enzymes in pteridine metabolism. With this information, we have developed a comprehensive model of the salvage and metabolism of pteridines in *Leishmania*.

MATERIALS AND METHODS

Cell Lines and Culture—All lines of *Leishmania* were derived from *L. major* strain LT252 clone CC-1 and cultured in M199 medium containing 10% fetal bovine serum (24). In this medium parasites grow as the promastigote form, which normally resides extracellularly within the gut of the sand fly insect vector. Null mutant *Leishmania* lacking DHFR-TS (*dhfr-ts*$^-$) or PTR1 (*ptr1*$^-$) were created by targeted disruption of both alleles of each gene (10, 25). The *ptr1*$^-$ mutant was grown with H_2- or H_4biopterin (2–4 μg/ml), and the *dhfr-ts*$^-$ mutant was grown with 10 μg/ml thymidine. The lines *ptr1*$^-$/+PTR1 and *dhfr-ts*$^-$/+DHFR-TS represent the respective null mutants transfected with plasmids pX63NEO-PTR1 (10) or pK300 (24) and overexpress PTR1 and DHFR-TS, respectively (Ref. 10; this work). In some experiments cells were grown in fdM199, which is M199 medium lacking folate and thymidine and supplemented with 0.66% bovine serum albumin (U. S. Biochemical Corp.) instead of serum. Pterin supplements were H_4biopterin (RBI), 6-hydroxymethylpterin, pterin, pteroic acid (Sigma), and a wide range of other pterins (Schircks Laboratories, Jona, Switzerland or from S. Kaufman, National Institutes of Health). H_2neopterin was prepared from neopterin by reduction with dithionite in the presence of ascorbate (26). Parasites were enumerated using a Coulter Counter (Model Zf) at the time when cultures grown in H_4biopterin had reached late log phase.

Expression and Purification of PTR1s—The initial steps of purification of recombinant *L. major* PTR1 have been described (10) and included expression in *Escherichia coli* using the pET-3a expression vector (27), induction, cellular lysis, and purification by ammonium sulfate precipitation and DEAE-cellulose chromatography. PTR1-containing fractions from the DEAE step were pooled and the buffer changed to 20 mM Mes, pH 6.0, by passage over PD10 columns of Sephadex G-25 (Pharmacia Biotech Inc.). Subsequent purification steps were carried out by fast protein liquid chromatography (Pharmacia). Protein was applied to an ion exchange Mono-S HR 5/5 column and eluted with a 20-min 0–0.2 M NaCl gradient at 1 ml/min. An ion exchange Mono-Q 5/5 column was also tested and found to give an equivalent purification. PTR1-containing fractions were combined, and the volume reduced to 1 ml using YM10 filters (Amicon). The concentrate was applied to a Superdex 200HR 10/30 column and eluted at a flow rate of 0.5 ml/min with 20 mM Mes, pH 6.0, containing 0.1 M NaCl. Recombinant PTR1 was purified 10-fold with overall yields of 80%.

The coding region for *L. tarentolae PTR1* was amplified by the polymerase chain reaction using *Taq* polymerase, template DNA from the MG strain of *L. tarentolae*, and the primers SMB-8 (5′-ggcagatcTCAGGCCCGGGTAAGGC) and SMB-9 (5′-cgcagatctcccatATGACGACTTCTCCGA; lowercase letters indicate bases not present in *PTR1*), with 25 amplification cycles of 1 min at 94 °C, 1 min at 57 °C, and 2 min at 72 °C. The expected fragment was obtained, digested with *Nde*I and *Bgl*II, inserted into the pET-3a expression vector (Novagen), and transformed into *E. coli* strain BL21(DE3)/pLysS (27). The expression of *L. tarentolae* PTR1 was induced and the enzyme purified as described for *L. major*.

Native PTR1 was purified from 7.5×10^{10} *ptr1*$^-$/+PTR1 *L. major*, in a manner similar to that used for the recombinant enzyme except that the cells were lysed by 3 cycles of freezing and thawing followed by sonication. The lysate was centrifuged at 100,000 $\times$ *g* for 30 min, and the supernatant was loaded onto a DEAE-cellulose column, eluted (10), and further purified as described for the recombinant enzyme. Native PTR1 was purified 200-fold and obtained in 72% yield. Purified PTR1 preparations were stored at −80 °C in the presence of 20% glycerol and 20 mM β-mercaptoethanol.

Gel Filtration Chromatography—The molecular weights of nondenatured PTR1s were estimated on a Sephacryl S-200 column (120 $\times$ 0.8 cm) at a flow rate of 0.5 ml/min. Three different pH values were tested using the following buffers: 20 mM Tris-HCl, pH 7.0, 20 mM $NaPO_4$, pH 6.0, or 20 mM sodium acetate, pH 4.7, each containing 0.1 M NaCl. Molecular mass markers were β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), carbonic anhydrase (29 kDa), and cytochrome *c* (12.4 kDa). Fractions were monitored at 280 nm and for PTR1 activity.

Enzymatic Assays—Spectrophotometric pteridine reductase assays were performed at 30 °C in the presence of NADPH (usually 100 μM) and pteridines as indicated (10). The pH dependence of PTR1 activity was determined using three overlapping buffers: 20 mM sodium acetate, pH 3.6–6.0, $NaPO_4$, pH 5.5–7.5, or Tris-HCl, pH 7.0–8.0. Radiometric assays of folate and/or H_2folate reductase activities (28) were performed using 40 μM [3′,4′,7,9-^{3}H]folate (24.1 Ci/mmol, Moravek Biochemicals), which was purified prior to use (29). To test the nature of the product formed from reduction of biopterin or H_2biopterin by PTR1 or DHFR-TS, a coupled assay was used (30) where the synthesis of H_4biopterin is linked to the hydroxylation of [4-^{3}H]Phe (27 Ci/mmol, Amersham Corp.) by mammalian phenylalanine hydroxylase (Sigma). After incubation for 30 min at 25 °C, the [^{3}H]Tyr formed was iodinated, the sample was passed over a Dowex 50 column, and the tritiated water was quantified by scintillation counting.

Enzyme Kinetics and Inhibitor Studies—The kinetic parameters K_m and V_{max} for the pteridine substrates were measured in a spectrophotometric assay with 100 μM NADPH as described previously (10). Extinction coefficients used for various pteridines were determined spectrophotometrically, and PTR1 activity was calculated based on the decrease in absorbance of both NADPH and the pteridine substrates. Kinetic data for oxidized pteridines were evaluated by fitting to the Michaelis-Menten equation by nonlinear regression (Hyper Version 1.02A; J.S. Eastby, Liverpool, UK). Both H_2folate and H_2biopterin showed substrate inhibition at concentrations above 5 and 10 μM, respectively, and for these, K_m, V_{max}, and K_i (for substrate) values were evaluated using graphical plots and the general equation for substrate inhibition (31). For inhibition studies, PTR1 was incubated with MTX and NADPH and the reaction initiated with the pteridine substrate (40 μM folate, 100 μM biopterin, 10 μM H_2biopterin, or 5 μM H_2folate). Inhibition was examined at several concentrations of enzyme, and the data were analyzed using a method for tight binding inhibitors to obtain K_i (32).

Purification and Assay for DHFR-TS—Recombinant DHFR-TS from *L. major* was purified from a *dhfr*$^-$ *E. coli* strain (33) bearing the expression plasmid 02CLSA-4 (34). Cells were lysed by two cycles through a French press (15,000 p.s.i.), and DHFR-TS was purified by binding and elution from a MTX-Sepharose column (Sigma) (34, 35). The eluate was concentrated using YM10 membrane filters (Amicon) and loaded onto a Sephacryl S-200 column (120 $\times$ 0.8 cm). Electrophoretically homogeneous enzyme was eluted with 50 mM Tris·HCl, 0.1 M NaCl at a flow rate of 0.5 ml/min, desalted over PD10 columns of Sephadex G-25 (Pharmacia), and stored at −80 °C in the presence of 10% glycerol.

Antibodies to PTR1 and Western Blot Analysis—Polyclonal antiserum against PTR1 was elicited in New Zealand White rabbits using 200 μg of *L. major* PTR1 in Freund's complete adjuvant (Sigma) in the primary immunization. The rabbits were boosted 5 times with 100 μg PTR1 each in incomplete Freund's adjuvant at 3-week intervals, and serum was obtained after the last bleeding. For immunoblots, purified PTR1 and crude *Leishmania* extracts were separated on a 12.5% SDS-polyacrylamide gel (36) and electrophoretically transferred onto Millipore polyvinylidene difluoride membranes (37) using a semi-dry blot apparatus (Owl Scientific). Blots were incubated with antiserum to

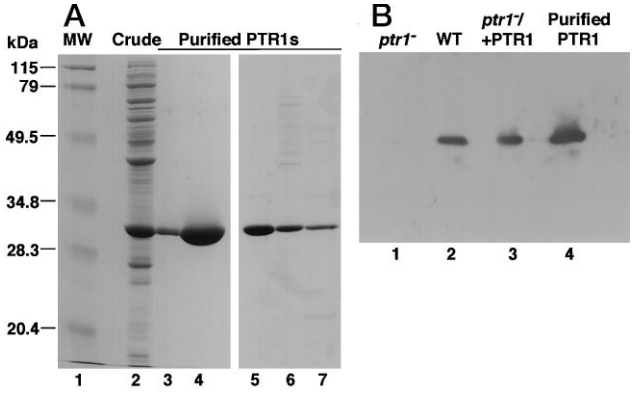

FIG. 1. **Purification and analysis of PTR1 by Western blotting.** *A*, proteins were separated on 12.5% acrylamide gels and stained with Coomassie Blue. *Lane 1*, molecular mass markers; *lane 2*, 35–55% ammonium sulfate precipitate of crude extracts of *E. coli* expressing *L. major* PTR1 (50 μg); *lanes 3* and *4*, purified recombinant *L. major* PTR1 after chromatography on Superdex HR 200 column (5 and 50 μg, respectively). *Lanes 5–7*, recombinant *L. major* (5 μg), native *L. major* (5 μg), and recombinant *L. tarentolae* (2.5 μg) PTR1, respectively, from fast protein liquid chromatography mono S column. *B*, total cellular or purified proteins electrophoresed on SDS-polyacrylamide gels were blotted as described under "Materials and Methods." *Lane 1*, *ptr1*$^-$ (100 μg); *lane 2*, wild-type (100 μg); *lane 3*, *ptr1*$^-$/+PTR1 (1 μg); and *lane 4*, purified recombinant *L. major* PTR1 (0.1 μg).

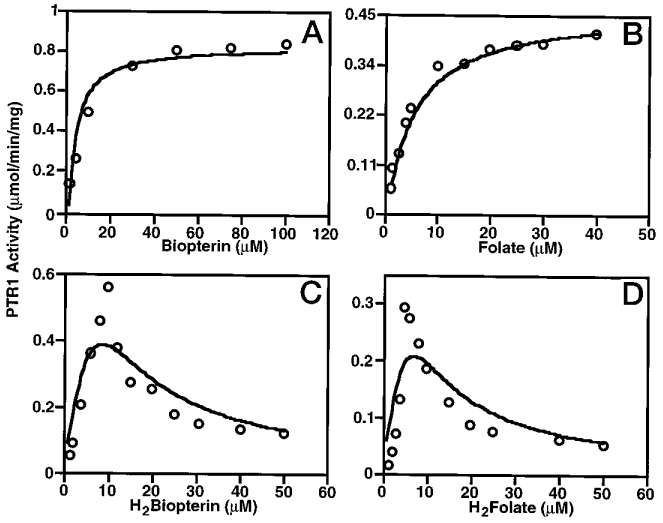

FIG. 2. **Substrate inhibition of PTR1 activity by H_2pteridines.** PTR1 activity was assayed with recombinant *L. major* enzyme using 20 mM sodium acetate, pH 4.7 (biopterin and H_2biopterin), or 20 mM $NaPO_4$, pH 6.0 (folate and H_2folate). *A*, biopterin; *B*, folage; *C*, H_2biopterin; and *D*, H_2folate. The *curves* shown in *A* and *B* were calculated assuming Michaelis-Menten kinetics, and the *curves* shown in *C* and *D* were calculated using the general equation for substrate inhibition (31) with the values shown in Table I.

PTR1 (1:1000), and binding was detected using either horseradish peroxidase-conjugated goat anti-rabbit antibody (1:3000) and chemiluminescence (Amersham Corp.) or alkaline phosphatase-conjugated goat anti-rabbit antibody and developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium.

Preparation of Crude Leishmania Extracts—Late logarithmic phase promastigotes were collected by centrifugation, washed twice with phosphate-buffered saline (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.8 mM KH_2PO_4), and resuspended (3×10^9/ml) in phosphate-buffered saline supplemented with 1 mM EDTA and a mixture of protease inhibitors suggested by Meek *et al.* (20). Cells were lysed by freeze thawing and sonication and the extracts clarified by centrifugation at 15,000 $\times$ *g* for 30 min.

RESULTS

Purification of PTR1s—Previously we reported upon the partial purification of *L. major* PTR1, expressed in engineered *E. coli* (10). Inclusion of two additional steps (ion exchange and gel filtration chromatography) yielded preparations that were electrophoretically homogeneous, even when the gel was overloaded (Fig. 1*A, lanes 3–5*). We also overexpressed and purified native PTR1 from *L. major* parasites and recombinant *L. tarentolae* PTR1. The recombinant and native PTR1s behaved similarly during purification and exhibited similar mobilities upon SDS-polyacrylamide gel electrophoresis (Fig. 1*A, lanes 5–7*). The apparent subunit molecular masses were 30 kDa (10, 11, 16, 17).

Western blot analysis with a polyclonal antiserum to recombinant *L. major* PTR1 detected a 30-kDa protein in wild-type *L. major* extracts whose size was identical to that of purified PTR1s (Fig. 1*B, lanes 2–4*). This protein was absent in the *ptr1*$^-$ *L. major* deletion mutant obtained previously by gene targeting (Fig. 1*B, lane 1*) (10) and was expressed at approximately 100-fold higher levels in the *L.major* line overexpressing PTR1 (Fig. 1*B, lane 3*; note that 100-fold less protein was loaded in *lane 3*). In wild-type cells, PTR1 constituted about 0.01% of the total cellular protein.

By gel filtration chromatography, the apparent molecular mass of PTR1 was estimated to be 116 and 117 kDa for the recombinant and native *L. major* enzymes, respectively (not shown). Similar values were obtained at pH values of 4.7, 6.0, and 7.0 (data not shown). We infer that PTR1 is a tetramer of identical 30-kDa subunits and that significant alterations in molecular shape are not associated with differences in the pH dependence of folate *versus* biopterin reduction (below).

Enzymatic Properties of PTR1—Previous studies of partially purified PTR1 showed it to have two pH optima, one of about 4.7 for biopterin and H_2biopterin and one of about 6.0 for folate and H_2folate (10). Studies of the homogeneous *L. major* and purified *L. tarentolae* PTR1s have refined and extended these initial findings.

At the optimum pH for each substrate, PTR1 activity with oxidized biopterin and folate exhibited standard Michaelis-Menten kinetics (Fig. 2). However, H_2biopterin and H_2folate showed substrate inhibition at concentrations above 10 and 5 μM, respectively (Fig. 2). V_{max} values with H_2biopterin and H_2folate were derived from analyses that included considerations of substrate inhibition (31) and yielded values that were at least 50% that of the corresponding oxidized pteridines (Table I). Previously, only substrate concentrations of 100 μM were tested (10), which led to a 3–4-fold underestimate of the rate of reduction of H_2pteridines by PTR1. H_2neopterin and H_2sepiapterin also showed substrate inhibition, whereas L- and D-biopterin, L- and D-neopterin, 6-hydroxymethylpterin, L- and D-monapterin, 6-formylpterin, and 6,7-dimethylpterin showed standard Michaelis-Menten kinetics (data not shown). This suggests that substrate inhibition was a general feature of PTR1 activity, but only with H_2pteridines.

We then examined the pH dependence of PTR1 activity. With biopterin a sharp peak of activity was observed at pH 4.7 (Fig. 3*A*). Activity with H_2biopterin was also optimal at pH 4.7, although the peak was somewhat less sharp (Fig. 3*B*). A pH optimum of 4.7 was found for PTR1 activity with every pterin tested (L- and D-biopterin, L- and D-neopterin, 6-hydroxymethylpterin, L- and D-monapterin, 6-formylpterin, 6, 7-dimethylpterin, H_2sepiapterin; data not shown). In contrast, with folate maximal activity occurred at pH 6.0 (Fig. 3*C*), and with H_2folate a broad pH optimum was found, from about 5 to 7.5 (Fig. 3*D*). Thus, pH optima criteria divide PTR1 substrates into pterins *versus* folates, rather than by oxidation state as observed for substrate inhibition.

Based on this information, we determined the kinetic properties for the recombinant and native *L. major* PTR1s, and *L.*

TABLE I
Kinetic parameters for Leishmania PTR1s

Results are the average of 2–4 determinations presented with standard deviations.

Pteridine substrate	pH[a]	K_m (pteridine)	K_m (NADPH)	K_i[b] (pteridine)	V_{max}	K_i (MTX)
		μM	μM	μM	μmol/min/mg	nM
Recombinant *L. major* PTR1						
Biopterin	4.7*	12.2 ± 1.6	13.2 ± 0.6	NI[c]	1.2 ± 0.3	30.7 ± 5.7
	6.0	19.8 ± 2.5	ND	NI[c]	0.66 ± 0.07	102 ± 25
	7.0	39.9 ± 5.9	ND	NI[c]	0.21 ± 0.03	276 ± 61
H_2 biopterin	4.7*	7.6 ± 2.8	14.5 ± 1.8	14.5 ± 0.9	0.87 ± 0.2	58.3 ± 15
	6.0	5.6 ± 1.7	ND	21.2 ± 3.5	0.50 ± 0.09	88 ± 20
	7.0	5.4 ± 2.3	ND	18.2 ± 2.7	0.23 ± 0.04	342 ± 110
Folate	4.7	1.6 ± 0.3	ND	NI[c]	0.32 ± 0.05	200 ± 23
	6.0*	2.6 ± 0.4	12.2 ± 0.9	NI[c]	0.56 ± 0.2	265 ± 28
	7.0	8.5 ± 3.4	ND	NI[c]	0.29 ± 0.04	801 ± 172
H_2folate	4.7	6.1 ± 1.0	ND	13.1 ± 1.3	0.22 ± 0.06	176 ± 37
	6.0*	3.4 ± 0.2	14.2 ± 1.2	13.5 ± 0.9	0.38 ± 0.07	191 ± 50
	7.0	5.4 ± 1.2	ND	11.2 ± 2.5	0.25 ± 0.04	509 ± 185
Native *L. major* PTR1						
Biopterin	4.7*	10.1 ± 1.4	11.6 ± 1.1	NI[c]	0.55 ± 0.1	26.3 ± 4.7
Folate	6.0*	2.4 ± 0.3	13.5 ± 2.6	NI[c]	0.28 ± 0.1	ND
Recombinant *L. tarentolae* PTR1						
Biopterin	4.7*	10.9 ± 2.5	12.3 ± 1.7	NI[c]	0.98 ± 0.1	28.3 ± 8
H_2biopterin	4.7*	8.5 ± 2.4	9.35 ± 4.8	21.1 ± 2.7	0.62 ± 0.1	62.5 ± 22
Folate	6.0*	1.9 ± 0.3	14.6 ± 1.1	NI[c]	0.46 ± 0.1	248 ± 26
H_2folate	6.0*	6.7 ± 1.6	12.0 ± 5.5	21.5 ± 3.2	0.23 ± 0.02	210 ± 29

[a] Kinetic parameters were determined in 20 mM each of sodium acetate, pH 4.7, or sodium phosphate, pH 6.0 or 7.0 (as appropriate substrates were fixed at 100 μM NADPH; 100 μM biopterin; 40 μM H_2folate; 10 μM H_2biopterin; 5 μM H_4folate).
[b] K_i for pteridine substrates that exhibit inhibition of enzymatic activity.
[c] NI − PTR1 activity is not inhibited by substrate at maximum concentrations used for kinetic analysis (40–100 μM). * indicates optimum pH for each substrate. ND, not determined.

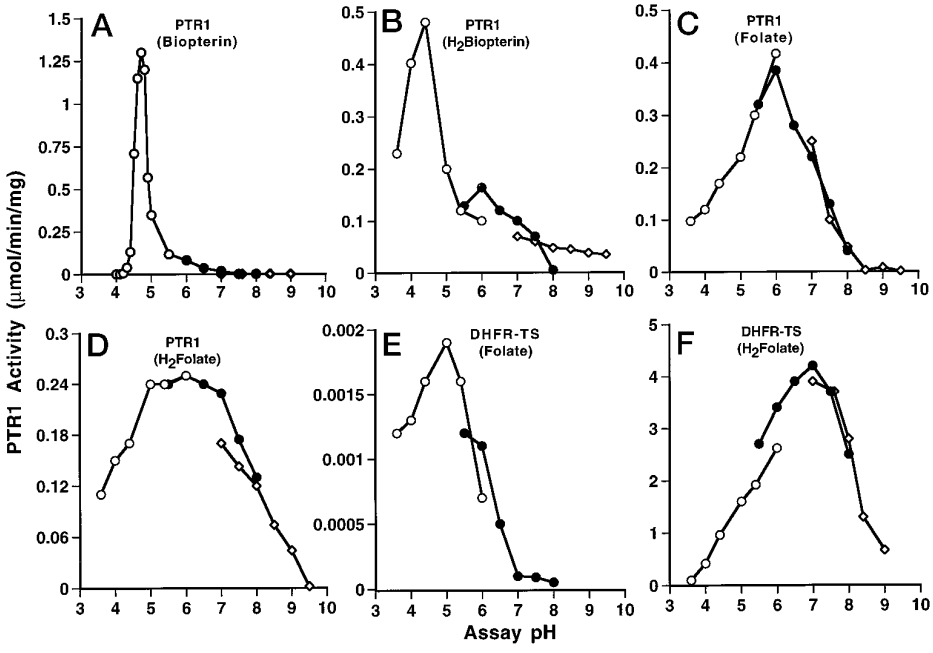

FIG. 3. **pH dependence of PTR1 and DHFR-TS activity.** Assays were performed with recombinant *L. major* PTR1 or DHFR-TS using three different overlapping buffers at the indicated pH. The buffers were ○, 20 mM (PTR1) or 50 mM (DHFR-TS) sodium acetate; ●, sodium phosphate; and ◇, Tris-HCl. DHFR-TS activity with folate was determined with the radiometric method, all other activities were determined with the spectrophotometric method.

tarentolae PTR1, at pH 4.7, 6.0, or 7.0 with biopterin, H_2biopterin, folate, and H_2folate (Table I). The properties of all three enzymes were very similar, showing first that the recombinant enzyme faithfully represented the native *L. major* enzyme and second that PTR1s from different species catalyze similar reactions.

For all PTR1s, the K_m for NADPH was 9–15 μM, and this was insensitive to enzyme source, pH, and substrate (Table I). At optimum pH values, biopterin displayed the highest K_m (10–12 μM); H_2biopterin and H_2folate were intermediate (3.4–8.5 μM), and folate had the lowest K_m (1.9–2.6 μM; Table I). For H_2biopterin and H_2folate, substrate inhibition K_i values of 11–21 μM were obtained, 2–4-fold above the K_m calculated for these substrates (Table I). In general these values were not strongly affected by pH. This suggests that the differences between pterins and folates, or oxidized and reduced pteridines, arise from factors involving interaction with the substrates themselves, rather than the assay conditions.

MTX was a potent inhibitor of the recombinant *L. major* and *L. tarentolae* PTR1s, with all pteridines and at different pH values (Table I). Using the method of Cha (32) to calculate the K_i for tight binding inhibitors at the optimum pH for each substrate, MTX inhibited PTR1 activity with biopterin most strongly (K_i = 30 nM), followed by H_2biopterin (K_i = 60 nM), H_2folate (K_i = 200 nM) and folate (K_i = 255 nM). For all substrates the K_i was higher at pH 7.0 than at optimal pH, show-

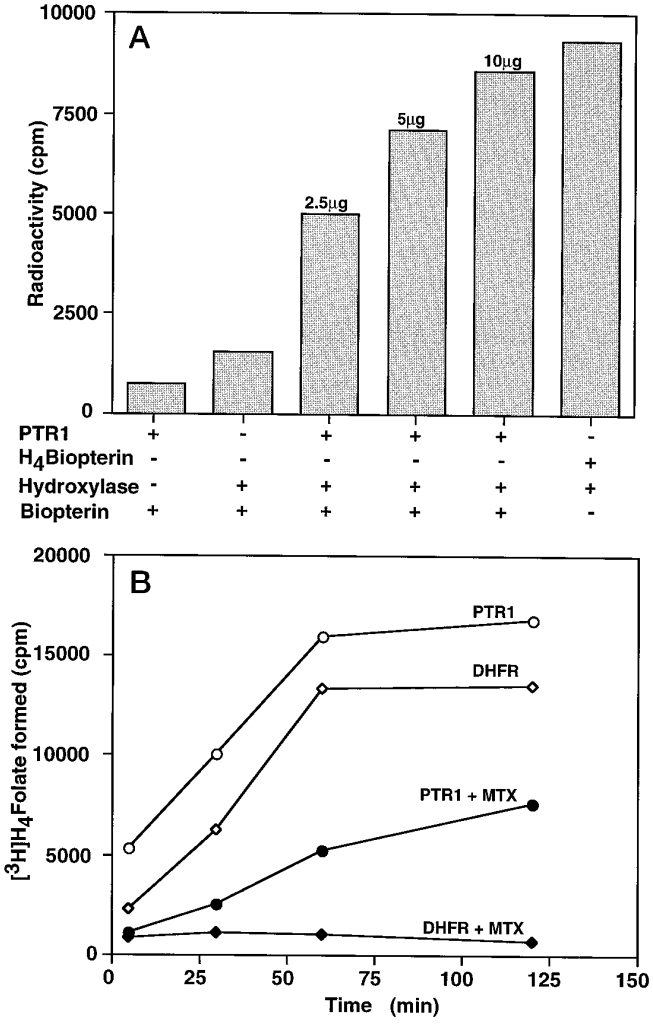

FIG. 4. **Products of biopterin and folate reduction by PTR1.** *A*, phenylalanine hydroxylase activity was determined using 50 mM potassium phosphate, pH 6.8, and other essential components of the system listed under "Materials and Methods." Recombinant *L. major* PTR1 was compared with H_4biopterin (10 μM), and results are presented as radioactivity (cpm) arising from iodination of [^{3}H]tyrosine following duplicate determinations. The *numbers above* the *bars* represent the amount of PTR1 used in the assay. *B*, recombinant *L. major* PTR1 or bovine DHFR (2 μg each) was incubated with 40 μM [^{3}H]folate at pH 6.0 (50 mM potassium phosphate) for up to 120 min in the presence (+MTX) or absence of MTX. Results represent the amount of [^{3}H]H_4folate formed (cpm).

ing an increase of 6–9-fold for biopterins and 3–4-fold for folates (Table I).

Products of Pteridine Reduction by PTR1—We determined whether the action of PTR1 on biopterin yielded the biologically active H_4biopterin by coupling this reaction to the H_4biopterin-dependent formation of tyrosine (Tyr) by mammalian phenylalanine hydroxylase (30). In the absence of phenylalanine hydroxylase or PTR1, little Tyr formation was observed (Fig. 4*A*). Addition of increasing amounts of PTR1 resulted in increasing Tyr synthesis, with 10 μg of PTR1 showing as much activity as 10 μM H_4biopterin (Fig. 4*A*; it should be noted that the conditions of this assay, pH 6.8, are not optimal for PTR1 activity). Similar results were obtained when biopterin was replaced with H_2biopterin in the assay mixture (not shown). Thus, PTR1 directs the synthesis of biologically active H_4biopterin, presumably the (6*R*)-L-*erythro*-5,6,7,8-H_4biopterin substrate of phenylalanine hydroxylase.

We next asked whether PTR1 activity generated H_4folate. A radiometric assay was used where folate and H_2folate but not

TABLE II
Comparison of PTR1 and DHFR-TS activities with different pteridines

Pteridine substrate	pH	*Leishmania* PTR1s[a]	pH	*L. major* DHFR-TS[b]
		nmol/min/mg		*nmol/min/mg*
Biopterin	4.7	550–1200	4.7–7.4	<0.1
	7.0	210		
H_2biopterin	4.7	620–870	4.7–7.4	<0.1
	7.0	230		
Folate	6.0	280–560	5.0	2.5 ± 0.9
	7.0	290		
H_2folate	6.0	280–380	7.0	6400 ± 110
	7.0	250		

[a] V_{max} values taken from Table I.
[b] V_{max} is shown. Activity was measured by the radiometric assay with folate and the spectrophotometric assay with H_2folate.

H_4folate were precipitated in the presence $ZnSO_4$ (28). By these criteria, recombinant *L. major* PTR1 mediated the formation of H_4folate from both folate (Fig. 4*B*) and H_2folate (not shown). As expected, activity with folate was inhibited partially by 1.25 μM MTX, whereas bovine DHFR was completely inhibited (Fig. 4*B*). Thus, we conclude that PTR1 mediates the synthesis of H_4pteridines (the biochemically active forms) starting from either oxidized or H_2pteridines.

Comparison of PTR1 and DHFR-TS Activities—The activities of PTR1 toward pterins and folates overlap those of DHFRs purified from various sources (Table I; Ref. 38). The activity of the *Leishmania* DHFR-TS enzyme with pterins or oxidized folate had not been reported, and we purified the *L. major* DHFR-TS from engineered *E. coli* (20, 34, 39). The recombinant enzyme prepared by these methods is known to exhibit the same properties as the native enzyme, when assayed with H_2folate or for TS activity (20, 34, 39).

DHFR-TS activity was optimal at pH 5.0 with folate (K_m = 4.1 ± 2.6 μM), and at pH 7.0 with H_2folate (Fig. 3, *E* and *F*). In contrast, PTR1 activity was maximal at pH 6.0 with both substrates (Fig. 3, *C* and *D*). Relative to PTR1, DHFR-TS activity was 20-fold greater with H_2folate and 100-fold less with folate (Table II). We were unable to detect biopterin or H_2biopterin reduction by DHFR-TS in either spectrophotometric or coupled phenylalanine hydroxylase assays, at pH values from 4.7 to 7.4 (Table II; data not shown). Thus, *Leishmania* DHFR-TS has weak activity with folate and no detectable activity with pterin substrates.

PTR1 and DHFR-TS Activity in Leishmania Extracts—To determine the relative contributions of PTR1 and DHFR-TS to the reduction of folates in *L. major*, we measured activities in crude cellular extracts. We were aided by the availability of targeted null mutants lacking either the *PTR1* or *DHFR-TS* genes (10, 25), which permitted a genetic test of the contribution of each enzyme. Since nonspecific interference in crude extracts was high with the spectrophotometric assay, particularly at low pH, we used the radiometric assay with [^{3}H]folates at substrate concentrations yielding highest activity (Table I and Fig. 2).

H_2folate reduction was measured at pH 7, where both PTR1 and DHFR-TS exhibited high activity (Fig. 3, *D* and *F*). Comparisons of the wild-type, *ptr1*$^-$ (DHFR-TS only) and *dhfr-ts*$^-$ (PTR1 only) lines showed that more than 90% of cellular activity arose from DHFR-TS (Table III). The predominance of DHFR-TS agrees with the predicted relative contribution of these two enzymes, calculated from estimates of the cellular levels of these two proteins and their specific activities (Table II).

Folate reduction was measured at pH 5, 6, and 7; the data for pH 6 is shown in Table III. Since the radiometric assay follows

TABLE III

Observed and calculated contributions of PTR1 and DHFR-TS to folate and H_2folate reductase activities in Leishmania major crude extracts

Crude protein extracts were prepared from *Leishmania* harvested in the logarithmic phase of growth and pteridine reductase activities were measured radiometrically at pH 7.0 (H_2folate) and pH 6.0 (folate).

	Cell line				
	CC-1 (wild-type)	*ptr1*$^-$ (= DHFR-TS)	*dhfr-ts*$^-$ (= PTR1)	*ptr1*$^-$/+*PTR1*	Ratio DHFR-TS:PTR1
H_2folate reduction (nmol/min/mg)					
Observed	3.4 ± 0.7	3.1 ± 0.5	0.09 ± 0.05	4.3 ± 0.3	34
Calculated		3.6[a,b]	0.021[c,d]		171
Folate reduction (pmol/min/mg)					
Observed	18.7 ± 1.5	15.3 ± 2.2	2.3 ± 0.7	522 ± 52	7
Calculated		1.4[a,b]	56[a,d]		0.025

[a] Calculated using V_{max} values for *L. major* enzymes (Tables I and II).
[b] Calculated assuming DHFR-TS represents 0.056% of total cellular protein (20).
[c] Calculated using a specific activity of 208 nmol/min/mg with 5 μM H_2folate (Table I and Fig. 2).
[d] Calculated assuming PTR1 represents 0.01% of total cellular protein (Fig. 1*B*).

H_4folate rather than H_2folate formation (40), it is relevant to note that the H_2folate reductase activities of both PTR1 and DHFR-TS were comparable to or greater than that with folate (Fig. 3, *C–F*) and would thus not be limiting. As with H_2folate, most of the folate activity could be assigned to DHFR-TS, as the *ptr1*$^-$ mutant showed only an 18% reduction in activity, comparable to the 11% activity remaining in the *dhfr-ts*$^-$ mutant.

However, the predominance of DHFR-TS in folate reduction disagrees with that deduced from estimates of the cellular levels of these two proteins and their specific activities (Table II). We calculated that the contribution of PTR1 should be 40-fold higher than that of DHFR-TS, rather than 7-fold lower (Table III). This arises from discrepancies in both DHFR-TS and PTR1 activities, which were observed to be about 11-fold higher and 24-fold lower than calculated, respectively (Table III). To address this problem, we examined numerous different preparations and experimental conditions (varying pH and folate concentrations), verified that each assay was performed in the linear range of crude extract addition, and confirmed that radiometric and spectrophotometric assays yielded similar kinetic parameters with the purified enzymes (data not shown). None of these variables significantly altered the result shown in Table III. That the assay used could detect high levels of PTR1 is shown by studies of the *ptr1*$^-$/+PTR1 line, which shows a 200-fold increase in activity with folate (Table III), and by addition of purified PTR1 to the crude extracts, which yielded the expected activity (not shown). Last, mechanistic studies of purified PTR1 and/or DHFR-TS catalysis do not suggest an explanation for this observation (20).[2]

Leishmania Growth and PTR1 Activity with Diverse Pteridines—Leishmania are able to utilize a wide range of pteridines (8, 11), and we sought to establish whether PTR1, DHFR-TS, or possibly some other pteridine reductase was responsible for salvage. We utilized a folate-deficient medium (fdM199) in these studies to determine the ability of different pteridines to support the growth of wild-type or mutant *L. major* and compared these results with the relative activity of PTR1 with these substrates (Table IV). In fdM199 medium, supplementation with an active pteridine is required for growth, and this is not affected by provision of thymidine (which is required by the *dhfr-ts*$^-$ mutant).

Three different groups of oxidized pteridines emerged from these studies (Table IV). "Good" pteridine nutrients (L- and D-biopterin, 6-hydroxymethylpterin, L-neopterin) sustained the growth of wild-type *Leishmania* and were good PTR1 substrates (>59% the activity obtained with L-biopterin). PTR1 but not DHFR-TS was essential for growth with these pterins, as the *ptr1*$^-$ mutant failed to grow while the *dhfr-ts*$^-$ mutant grew normally. "Poor" pteridine nutrients (D-neopterin, L- and D-monapterin, 6,7-dimethylpterin, 6-formylpterin) failed to sustain growth of wild-type *Leishmania* but were able to support growth of the PTR1 overproducer. These pteridines showed reduced activity with PTR1, about 10–34% that of L-biopterin (Table IV). DHFR-TS overproduction failed to sustain growth with these nutrients, consistent with its lack of activity with pterin substrates (Table II, 4). Last, "inactive" pteridine nutrients (pterin, pteroic acid, xanthopterin, isoxanthopterin, 6-carboxypterin, and 7-biopterin) were unable to support growth of any *Leishmania* tested and, correspondingly, were weak or inactive PTR1 substrates (0- 6% the activity obtained with L-biopterin). Thus, the ability of oxidized pterins to sustain growth of *Leishmania* was correlated with their ability to serve as PTR1 substrates.

Several reduced pterins were also examined (Table IV). As expected, H_4biopterin supported growth in all lines. Remarkably, H_2biopterin and H_2neopterin also supported growth of the *ptr1*$^-$ mutant. Previously, this was attributed to the anticipated ability of DHFR-TS to reduce H_2biopterin; however, DHFR-TS lacks this activity (Table II). Last, H_2sepiapterin and H_4-6-methylpterin behaved as good pteridine nutrients in that they supported wild-type growth but, unlike the other H_2pteridines, failed to support growth of the *ptr1*$^-$ mutant.

DISCUSSION

Catalytic Properties of PTR1—We have purified and determined the enzymatic properties of recombinant and native PTR1 from *L. major* and recombinant PTR1 from *L. tarentolae*. These enzymes exhibited similar physical and catalytic properties, indicating that PTR1 does not undergo *Leishmania*-specific modification, and validating the use of the recombinant enzyme for more detailed studies. All PTR1s displayed good activity with both pterins (biopterin and others; Tables I, II, and IV) and folates (Tables I and II). However, there were significant variations in the catalytic properties among pteridine substrates, with PTR1 activity on pterins exhibiting a sharper, more acidic pH optimum relative to folates, and H_2pterins and H_2folate both showing significant substrate inhibition. The results also show that PTR1 is capable of reducing oxidized pteridines completely to the tetrahydro form.

The properties of PTR1 may be compared with other well-known pteridine reductases, such as DHFR and dihydropteridine reductase (DHPR). Although DHPR shows sequence similarities placing it in the "short chain dehydrogenase family" with PTR1 (16–19), PTR1 is more closely related to other members of this family and does not exhibit activity with "quinonoid" H_2biopterin (10). Conversely, DHPR does not exhibit activity with folates or H_2pterins, other than those in the quinonoid form (19, 41).

[2] J. Luba, personal communication.

TABLE IV
Growth of Leishmania and PTR1 activity

Leishmania lines were inoculated into fdM199 supplemented with 10 μg/ml thymidine and/or 5 μg/ml of each pteridine and enumerated after the 6th passage.

Pteridine supplements	Ability to support growth in defined medium[a]					Relative PTR1 activity
	Wild-type	*ptr1*$^{-}$	*ptr1*$^{-1}$ +PTR1	*dhfr-ts*$^{-1}$	*dhfr-ts*$^{-1}$ +DHFR-TS	
Good pterin nutrients						
L-Biopterin	96	0	98	103	83	100
D-Biopterin	95	0	75	75	84	59
6-Hydroxymethylpterin	146	0	117	83	82	98
L-Neopterin	76	0	95	85	97	76
Poor pterin nutrients[c]						
D-Neopterin	0	0	65	0	0	19
L-Monapterin	0	0	63	0	0	10
D-Monapterin	0	0	75	0	0	17
6,7-Dimethylpterin	0	0	80	0	0	34
6-Formylpterin	0	0	74	0	0	16
Inactive pterin nutrients						
Pterin	0	0	0	0	0	6
Pteroic acid	0	0	0	0	0	0
Xanthopterin	0	0	0	0	0	3
Isoxanthopterin	0	0	0	0	0	4
6-Carboxypterin	0	0	0	0	0	2
7-Biopterin	0	0	0	ND	ND	0.2
Reduced pterins						
7,8-H_2L-biopterin	116	109	110	124	108	65[d]
7,8-H_2L-neopterin	95	87	92	ND	ND	62[d]
7,8-H_2sepiapterin	89	0	96	121	111	13[d]
5,6,7,8-H_4L-biopterin	100	100	100	100	100	ND
6-Methyl-5,6,7,8-H_4pterin	114	0	89	89	86	ND

[a] Growth was measured as a percent relative to parasites propagated in H_4biopterin.
[b] Activity assayed with recombinant *L. major* PTR1 using the indicated oxidized pterins (100 μM) or H_2pterins (10 μM) in 20 mM sodium acetate, pH 4.7, and NADPH (100 μM). Activity is expressed as a percent of the rate with biopterin as substrate.
[c] Pteridines also tested for ability to support *Leishmania* growth at 20 μg/ml.
[d] Activity assayed at 10 μM because these pteridines exhibit PTR1 inhibition at higher concentrations.

DHFRs from various sources exhibit activity with both folates and pterins (42, 43) but, unlike PTR1, are much less active with folate than H_2folate. Substrate inhibition has been observed previously with folate and H_2folate with the *Lactobacillus casei* DHFR (44) and with a number of H_2pterins with rat DHFR (42). The latter finding was attributed to either a lack of reducing agents in the assay mix or the presence of inhibitory pterins such as biopterin. However, biopterin would not inhibit PTR1 nor did reductants affect the activity (data not shown). Substrate inhibition is thus an intrinsic property of PTR1, perhaps arising from allosteric interactions of tetrameric PTR1, or mechanisms described previously with other proteins (31). Substrate inhibition is often considered non-physiological since, when present, it often occurs at high substrate levels. Current data suggest that the intracellular levels of folates and biopterin are 2–20 μM in *Leishmania* (7, 8, 29), but the activities of PTR1 and DHFR would be expected to keep the levels of H_2pteridines low. When inhibited by the action of antifolates, H_2pteridine levels could rise to a point where substrate inhibition could be significant.

Although the substrate specificities of PTR1 resemble those of DHFRs from other species, differences in catalytic mechanism relative to that of DHFRs were evident in the pH dependence of PTR1 activity. Typically DHFRs display weak activity with folate that is optimal around pH 5, whereas much higher activity is observed with H_2folate with two pH optima around pH 5 and 7 (44–48). In contrast, PTR1 activity was comparable with folate and H_2folate, with a pH optimum around 6 (Fig. 3).

Despite its shared evolutionary ancestry with the short chain dehydrogenase family which includes DHPR, the properties of PTR1 have converged on those of DHFR, albeit with important catalytic differences. A similar process may have occurred independently with the prokaryotic type II DHFRs, which lack sequence or structural homology to chromosomal DHFRs (49). How PTR1 independently attained its role as a novel pteridine reductase is an interesting question in the evolution of catalytic pathways. Currently, we are pursuing studies of the detailed catalytic mechanism and three-dimensional structure of PTR1 in our effort to shed light on this process.

A Comprehensive Model for Pteridine Metabolism in Leishmania—Our findings have permitted us to develop a general model for pteridine metabolism in *Leishmania* (Fig. 5), which provides a convenient framework for evaluating current data and developing future studies. The evidence for this model, and its implications to pteridine metabolism and chemotherapeutic inhibition, is discussed below.

The Role of PTR1 in Pterin Salvage—We have tested and confirmed the proposal that PTR1 was responsible for salvage of oxidized pteridines (10) in several ways. First, the ability of *L. major* to grow on a wide range of oxidized pterins correlates well with their activity as PTR1 substrates (Table IV). Good PTR1 substrates support *Leishmania* growth, and poor substrates require elevated PTR1 levels to support growth. Notably, the most physiologically abundant pterins in mammals, neopterin and biopterin, are the best substrates for PTR1 activity and *Leishmania* growth, whereas insect pterins such as xanthopterin are inactive (Table IV). Our findings are also in good agreement with results presented previously for growth of *Leishmania donovani* (8) and growth and altered PTR1 expression in *L. tarentolae* (17, 50), suggesting that PTR1 plays the same role in all *Leishmania* species. Second, deletion of *PTR1*, but not *DHFR-TS*, resulted in loss of the ability to grow on oxidized pterins (Table IV). Third, DHFR-TS showed no activity with pterins such as biopterin (Table II) nor did overproduction of DHFR-TS alter the pterin growth profile of *Leishmania* (Table IV). Thus, PTR1 alone accounts for salvage of oxidized pterins in *Leishmania*.

The Relative Contributions of PTR1 and DHFR-TS to Pteridine Metabolism—Although DHFR-TS plays no role in the reduction of pterins, PTR1 possesses significant activity with

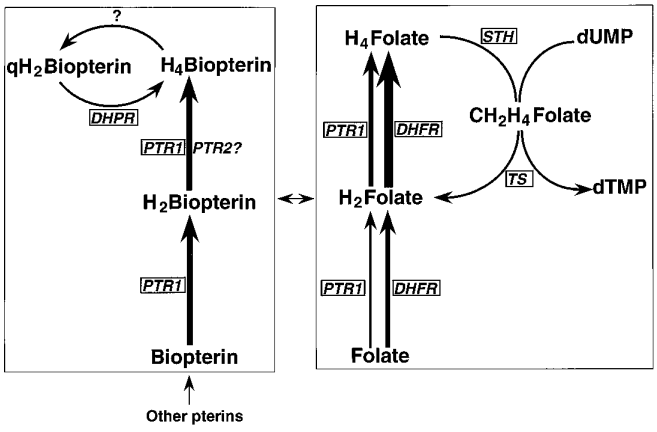

FIG. 5. **Proposed enzymatic pathways for the synthesis of reduced pteridines in *Leishmania*.** The width of the *arrows* indicate the relative contribution of each enzyme in steps where more than one is implicated. *STH*, serine transhydroxymethylase; *CH_2-H_4Folate*, 5,10-methylene tetrahydrofolate; *DHPR*, dihydropteridine reductase; *DHFR-TS*, dihydrofolate reductase-thymidylate synthase; *PTR1*, pteridine reductase 1; *PTR2*, hypothetical pteridine reductase; *?*, enzyme not known; qH_2Biopterin, quinonoid dihydrobiopterin.

folates (Tables I and II). By studying the reduction of folate and H_2folate in *Leishmania* crude extracts, from wild-type and lines lacking PTR1 or DHFR-TS, we were able to assess their relative contributions to pteridine metabolism. For H_2folate, more than 90% of the activity arose from DHFR-TS, a finding supported by calculations based upon the levels of PTR1 and DHFR-TS protein and their respective specific activities (Table III). However, for folate discrepant results were obtained. Comparisons of the null mutants suggested that more than 80% of the activity was contributed by DHFR-TS, whereas we calculated that 98% of this activity should arise from PTR1. We were unable to reconcile this difference, despite extensive testing and variation of experimental conditions, and it may reflect the existence of other activities not yet accounted for in our studies (below). Minimally, genetic deletion studies establish the dependence of the cellular folate reductase activity upon the presence of either PTR1 or DHFR-TS. For this reason, Fig. 5 depicts DHFR-TS as the major path of folate reduction within *Leishmania*.

What Is Responsible for Reduction of H_2biopterin?—The *ptr1*$^-$ mutant was shown to grow normally on H_2biopterin alone (Table IV) (10). Previously this was attributed to an expected H_2biopterin activity of DHFR-TS; however, we showed here that DHFR-TS lacks this activity (Table II). One explanation postulates the existence of an enzyme, "PTR2," possessing H_2biopterin but not biopterin reductase activity. An enzyme exhibiting activity with both H_2biopterin and H_2folate, but not biopterin and folate, has been described previously in the related trypanosomatid *Crithidia* (51, 52), and alternative pteridine reductases unrelated to either DHPR or DHFR have been detected in *E. coli* (53). Thus far, we have not been able to detect H_2biopterin reductase in crude preparations derived from *ptr1*$^-$ *L. major* (data not shown).

Interconversions of Pterins and Folates—A number of studies have demonstrated that the trypanosomatid growth requirement for folate can be reduced or even eliminated by inclusion of pterins such as biopterin (5–11) (Table IV). Although growth studies can be compromised by the presence of trace contaminants, incorporation of radiolabeled biopterin into folates has been shown in *L. donovani* (9), suggesting the occurrence of a *de novo* synthetic pathway. In contrast, another study failed to find incorporation of radiolabeled *para*-aminobenzoic acid into folate in *L. major* (54), which would be expected assuming that folates are synthesized by the classic route of dihydropteroate synthase. Most dihydropteroate synthase inhibitors are ineffective in *Leishmania* (55–57), and the few that are active show an independent, non-folate based mode of action (6). Thus, the mechanism of pterin/folate interconversion is not specifically indicated in Fig. 5.

What Is the Role (If Any) of Biopterin in Leishmania?—The role of biopterin in trypanosomatids is unknown. In other organisms, H_4biopterin plays a key role in the hydroxylation of phenylalanine and tyrosine, cleavage of ether-linked lipids, and the biosynthesis of nitric oxide (13, 14, 58, 59). However, trypanosomatids lack phenylalanine hydroxylase activity (60), and recently we have shown that ether-linked lipid cleavage uses NADPH rather than H_4biopterin as a cofactor (61). Thus, it is conceivable that *Leishmania* does not use H_4biopterin directly.

However, H_4biopterin has been demonstrated in the related trypanosomatid *Crithidia*, and *Crithidia* and *Leishmania* both possess DHPR activity, which in other organisms is responsible for recycling the quinonoid H_2biopterin formed by enzymatic use of H_4biopterin (Fig. 5) (10, 62). Second, improvements in defined media and methodology suggest that *L. major* is in fact unable to grow in the presence of folate alone and that previous results from our lab to the contrary reflect the occurrence of a pterin breakdown product in most folate preparations.[3] Moreover, neither folate nor H_2folate can rescue the growth defect of *ptr1*$^-$ *Leishmania* (10). Thus, pterins are required for *Leishmania* growth independently of their role in folate biosynthesis. Third, recently we have shown that PTR1 levels, by affecting the formation of reduced cellular biopterin, affect the sensitivity of *Leishmania* to oxidants.[3] Cumulatively, these data point to an essential role of H_4biopterin in *Leishmania* metabolism.

Role of PTR1 in MTX Resistance and Sensitivity to Antifolates—Amplification of the *Leishmania PTR1* gene within the H region is often observed in MTX-resistant *Leishmania* (reviewed in Refs. 50 and 63). The data in this work now provide a clear rationale for this process. As an alternative H_2folate reductase with 4000-fold less sensitivity to MTX than DHFR at physiological pH (500 *versus* 0.13 nM; Table I), PTR1 is poised to provide a metabolic "by-pass" of DHFR-TS inhibition (10). However, due to its weaker contribution (relative to DHFR-TS; Table III), PTR1 overexpression by gene amplification is apparently necessary to provide sufficient activity. Since the K_i for MTX inhibition is greater than 300 nM at pH 7 for all reactions performed by PTR1 (Table I), overexpression of any of these could also contribute to relieving inhibition of DHFR-TS, by increasing H_2folate pools indirectly through increased utilization of biopterin or directly by reduction of folate (Fig. 5).

The sensitivity of *Leishmania* to antifolates is dramatically affected (several orders of magnitude) by exogenous folate levels (6, 7, 10). For example, to show antifolate inhibition of the amastigote stage infecting macrophages, a folate-free medium was required (64). Modulation of antifolate inhibition also has been noted in the malaria parasite *Plasmodium falciparum* (65). In contrast, mammalian cells show relatively little effect and lack oxidized pteridine reductase activity (51, 66). Under conditions where DHFR-TS is inhibited, the ability of PTR1 in wild-type *Leishmania* to synthesize reduced folates could play a significant role in the modulation of MTX potency. Consistent with this, *ptr1*$^-$ *Leishmania* show hypersensitivity to MTX (10, 11).

Thus, for reasons both genetic and biochemical, future strategies oriented toward antifolate inhibition of *Leishmania*

[3] B. Nare and S. M. Beverley, manuscript in preparation.

should include inhibition of PTR1. In this regard, we have identified an inhibitor which shows good potency against both DHFR-TS and PTR1 activities, as well as *Leishmania* promastigote and amastigote growth, in medium containing physiological folate levels.[3] The principles established here promise to lead to improved chemotherapeutic inhibition of this important parasite, and in the future we hope to incorporate insights garnered from the three-dimensional structures of both DHFR-TS (34) and PTR1 in the search for clinically effective anti-parasite agents targeting this pathway.

In summary, improved understanding of the properties and roles of PTR1 and DHFR-TS in pteridine metabolism has permitted the establishment of a comprehensive model incorporating current knowledge of pteridine metabolism in *Leishmania*. This model provides a useful framework for formulating and testing new hypotheses of pterin metabolism and has led to an increased understanding of the question of antifolate inhibition and chemotherapy of *Leishmania*.

Acknowledgments—We thank Dr. Chen-Chen Kan of Agouron Pharmaceutical Inc. for advice, members of the Walsh lab at Harvard Medical School for helpful discussions on kinetic parameters of PTR1, and James Luba for pointing out the occurrence of substrate inhibition with H_2pteridines. We also thank Alexandre Bello for assistance with *Leishmania* culture in defined media and heterologous expression of PTR1 and D. Dobson, L. Epstein, L. A. Garraway, F. Gueiros-Filho, D. Kwon, and J. Moore for critical comments on the manuscript.

REFERENCES

1. Report of a WHO Expert Committee (1984) *The Leishmaniasis,* WHO Technical Report, Series 701, pp. 3–140.
2. Scott, D. A., Coombs, G. H., and Sanderson, B. E. (1987) *Mol. Biochem. Parasitol.* **23,** 139–149
3. Berman, J. D. (1988) *Rev. Infect. Dis.* **10,** 560–586
4. Nathan, H. A., Hutner, S. H., and Levin, H. L. (1956) *Nature* **178,** 741–743
5. Trager, W. (1969) *J. Protozool.* **16,** 372–375
6. Petrillo-Peixoto, M., and Beverley, S. M. (1987) *Antimicrob. Agents Chemother.* **31,** 1575–1578
7. Kaur, K., Coons, T., Emmett, K., and Ullman, B. (1988) *J. Biol. Chem.* **263,** 7020–7028
8. Beck, J. T., and Ullman, B. (1990) *Mol. Biochem. Parasitol.* **43,** 221–230
9. Beck, J. T., and Ullman, B. (1991) *Mol. Biochem. Parasitol.* **49,** 21–28
10. Bello, A. R., Nare, B., Freedman, D., Hardy, L., and Beverley, S. M. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 11442–11446
11. Papadopoulou, B., Roy, G., Mourad, W., Leblanc, E., and Ouellette, M. (1994) *J. Biol. Chem.* **269,** 7310–7315
12. Blakley, R., and Benkovic, S. J. (eds) (1985) *Folates and Pterins,* Vol. 2, pp. 1–399, John Wiley & Sons, Inc., New York
13. Tietz, A., Lindberg, M., and Kennedy, E. P. (1964) *J. Biol. Chem.* **239,** 4081–4090
14. Tayeh, M. A., and Marletta, M. A. (1989) *J. Biol. Chem.* **264,** 19654–19658
15. Kwon, N. S., Nathan, C. F., and Stuehr, D. J. (1989) *J. Biol. Chem.* **264,** 20496–20501
16. Callahan, H. L., and Beverley, S. M. (1992) *J. Biol. Chem.* **267,** 24165–24168
17. Papadopoulou, B., Roy, G., and Ouellette, M. (1992) *EMBO J.* **11,** 3601–3608
18. Krozowski, Z. (1994) *J. Steroid Biochem. Mol. Biol.* **51,** 125–130
19. Whiteley, J. M., Xuong, N. H., and Varughese, K. I. (1993) *Adv. Exp. Med. Biol.* **338,** 115–121
20. Meek, T. D., Garvey, E. P., and Santi, D. V. (1985) *Biochemistry* **24,** 678–686
21. Garrett, C. E., Coderre, J. A., Meek, T. D., Garvey, E. P., Claman, D. M., Beverley, S. M., and Santi, D. V. (1984) *Mol. Biochem. Parasitol.* **11,** 257–265
22. Beverley, S. M., Ellenberger, T. E., and Cordingley, J. S. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 2584–2588
23. Lazar, G., Zhang, H., and Goodman, H. M. (1993) *Plant J.* **3,** 657–668
24. Kapler, G. M., Coburn, C. M., and Beverley, S. M. (1990) *Mol. Cell. Biol.* **10,** 1084–1094
25. Cruz, A., Coburn, C. M., and Beverley, S. M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 7170–7174
26. Futterman, S. (1957) *J. Biol. Chem.* **228,** 1031–1038
27. Studier, F. W., Rosenberg, A. H., Dunn, J. J., and Dubendorff, J. W. (1990) *Methods Enzymol.* **185,** 60–89
28. Rothenberg, S. P., Iqbal, M. P., and Da Costa, M. (1980) *Anal. Biochem.* **103,** 152–156
29. Ellenberger, T. E., and Beverley, S. M. (1987) *J. Biol. Chem.* **262,** 10053–10058
30. Guroff, G., Rhoads, C. A., and Abramowitz, A. (1967) *Anal. Biochem.* **21,** 273–8
31. Cleland, W. (1979) *Methods Enzymol.* **63,** 501–513
32. Cha, S. (1975) *Biochem. Pharmacol.* **24,** 2177–2185
33. Howell, E. E., Foster, P. G., and Foster, L. M. (1988) *J. Bacteriol.* **170,** 3040–3045
34. Knighton, D. R., Kan, C. C., Howland, E., Janson, C. A., Hostomska, Z., Welsh, K. M., and Matthews, D. A. (1994) *Nat. Struct. Biol.* **1,** 186–194
35. Coderre, J. A., Beverley, S. M., Schimke, R. T., and Santi, D. V. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 2132–2136
36. Laemmli, U. K. (1970) *Nature* **227,** 680–685
37. Towbin, H., Staehlin, T., and Gordon, J. (1979) *Proc. Natl. Acad. Sci. U. S. A.* **76,** 4350–4354
38. Blakley, R. L. (1984) *Folates and Pterins* (Blakley, R. L., and Benkovic, S. J., eds) Vol. 1, pp. 191–253, John Wiley & Sons, New York
39. Grumont, R., Washtien, W. L., Caput, D., and Santi, D. V. (1986) *Proc. Natl. Acad. Sci. U. S. A.* **83,** 5387–5391
40. Hayman, R., McGready, R., and Van der Weyden, M. B. (1978) *Anal. Biochem.* **87,** 460–465
41. Shiman, R. (1985) in *Folates and Pterins* (Blakley, R. L., and Benkovic, S. J., eds) Vol. 2, 179–249, John Wiley & Sons, Inc., New York
42. Webber, S., and Whiteley, J. M. (1985) *Arch. Biochem. Biophys.* **236,** 681–690
43. Smith, G. K., Banks, S. D., Bingham, E. C., and Nichol, C. A. (1987) *Arch. Biochem. Biophys.* **254,** 416–420
44. McIntyre, L. J., and Harding, N. G. L. (1977) *Biochem. Biophys. Acta* **482,** 261–271
45. Blakley, R. L. (1984) in *Folates and Pterins* (Blakley, R. L., and Benkovic, S. J., eds) Vol. 1, pp. 191–253, John Wiley & Sons, Inc., New York
46. Zakrzewski, S. F., and Nichol, C. A. (1960) *J. Biol. Chem.* **235,** 2984–2988
47. Jarabak, J., and Bachur, N. R. (1971) *Arch. Biochem. Biophys.* **142,** 417–425
48. Mathews, C. K., and Huennekens, F. M. (1963) *J. Biol. Chem.* **238,** 3436–3442
49. Matthews, D. A., Smith, S. L., Baccanari, D. P., Burchall, J. J., Oatley, S. J., and Kraut, J. (1986) *Biochemistry* **25,** 4194–4204
50. Borst, P., and Ouellette, M. (1995) *Annu. Rev. Microbiol.* **49,** 427–460
51. Oe, H., Kohashi, M., and Iwai, K. (1983) *Agric. Biol. Chem.* **47,** 251–258
52. Oe, H., Kohashi, M., Matsuura, S., and Iwai, K. (1983) *Agric. Biol. Chem.* **47,** 425–427
53. Vasudevan, S. G., Paal, B., and Armarego, W. L. F. (1992) *Biol. Chem. Hoppe-Seyler* **373,** 1067–1073
54. Kovacs, J. A., Allegra, C. J., Beaver, J., Boarman, D., Michelle, L., Parillo, J. E., Chabner, B., and Masur, H. (1989) *J. Infect. Dis.* **160,** 312–320
55. Mattock, N. M., and Peters, W. (1975) *Ann. Trop. Med. Parasitol.* **69,** 359–370
56. El-On, J., Jacobs, G. P., Witztum, E., and Greenblatt, C. L. (1984) *Antimicrob. Agents Chemother.* **31,** 1575–1578
57. Neal, R. A. (1984) *Exp. Parasitol.* **57,** 269–273
58. Kaufman, S. (1963) *Proc. Natl. Acac. Sci. U. S. A.* **50,** 1085–1093
59. Kosar-Hashemi, B., and Armarego, W. L. F. (1993) *Biol. Chem. Hoppe-Seyler* **374,** 9–25
60. Kaufman, S. (1986) in *Chemistry and Biology of Pteridines 1986: Pteridines and Folic Acid Derivatives* (Cooper, A. A., and Whitehead, V. M., eds) pp. 185–200, Walter de Gruyter & Co., Berlin
61. Ma, D., Beverley, S. M., and Turco, S. J. (1996) *Biochem. Biophys. Res. Commun.* **227,** 885–889
62. Hirayama, K., Nakanisi, N., Sueoka, T., Katoh, S., and Yamada, S. (1980) *Biochim. Biophys. Acta* **612,** 337–343
63. Beverley, S. M. (1991) *Annu. Rev. Microbiol.* **45,** 417–444
64. Sirawaraporn, W., Sersrivanich, R., Booth, R. G., Hansch, C., Neal, R. A., and Santi, D. V. (1988) *Mol. Biochem. Parasitol.* **31,** 79–86
65. Milhous, W. K., Weatherly, N. F., Bowdre, J. H., and Desjardins, R. E. (1985) *Antimicrob. Agents Chemother.* **27,** 525–530
66. McDonald, J. D., and Bode, V. C. (1988) *Pediatr. Res.* **23,** 63–67